Molecular comparison of topotypic specimens confirms *Anopheles* (*Nyssorhynchus*) *dunhami* Causey (Diptera: Culicidae) in the Colombian Amazon

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The presence of Anopheles (Nyssorhynchus) dunhami *Causey in Colombia (Department of Amazonas) is confirmed for the first time through direct comparison of mtDNA cytochrome c oxidase I (COI) barcodes and nuclear rDNA second internal transcribed spacer (ITS2) sequences with topotypic specimens of* An. dunhami *from Tefé, Brazil.* An. dunhami *was identified through retrospective correlation of DNA sequences following misidentification as* Anopheles nuneztovari *s.l. using available morphological keys for Colombian mosquitoes. That* An. dunhami *occurs in Colombia and also possibly throughout the Amazon Basin, is of importance to vector control programs, as this non-vector species is morphologically similar to known malaria vectors including* An. nuneztovari, Anopheles oswaldoi *and* Anopheles trinkae. *Species identification of* An. dunhami *and differentiation from these closely related species are highly robust using either DNA ITS2 sequences or COI DNA barcode. DNA methods are advocated for future differentiation of these often sympatric taxa in South America.*

Key words: *Anopheles dunhami* - COI barcodes - ITS2 - topotypic - Brazil - Colombia

Anopheles (*Nyssorhynchus*) *dunhami* Causey was first described from Tefé, Amazonas (AM), Brazil (Causey 1945), but was placed in synonymy with the malaria vector *Anopheles nuneztovari* Gabaldón (Lane 1953), where it remained for some decades until taxonomic revisions resulted in its re-elevation (Peyton 1993). In the same study, Peyton also reduced the widespread east-Andean taxon *Anopheles trinkae* Faran to a junior synonym of *An. dunhami*, based on similarities in male genitalia (Peyton 1993). Later integrated studies, combining egg morphology, cytogenetics and rDNA sequences of the second internal transcribed spacer (ITS2), confirmed that *An. trinkae* and *An. dunhami* were indeed separate taxa and *An. trinkae* was reinstated to species status (Lounibos et al. 1998). Recently, Calado et al. (2008) reaffirmed the differences between *An. dunhami* and *An. nuneztovari* using the morphology of male genitalia and DNA sequences of the ITS2 and a portion of the mitochondrial cytochrome c oxidase I (COI). Overlapping morphological characters between proven malaria vectors *An. nuneztovari*, *An. trinkae* and *Anopheles oswaldoi* (Hayes et al. 1987, Olano et al. 2001, Quiñones et al. 2006, Gutiérrez et al. 2009) and isomorphic non-vector species such as *An. dunhami* continue to hamper correct species identification in South America and can impact the effectiveness of vector control programs.

As a result of its historically confused taxonomic status, few verified distribution records exist for *An. dunhami*. Records to date are limited to AM: Tefé (type locality) (Causey 1945, Peyton 1993), Tabatinga (Lounibos et al. 1998) and Parintins (Calado et al. 2008). Although Tabatinga is adjacent to the Colombian border, *An. dunhami* has never been reported from Colombia and the species is not included in the available morphological keys to Colombian taxa (Suárez et al. 1988, González & Carrejo 2007). Herein, we molecularly characterize topotypic *An. dunhami* using COI barcodes [after Hebert et al. (2003)] and ITS2 rDNA sequences and, through molecular correlation of sequence data, confirm the presence of *An. dunhami* in the Colombian Amazon for the first time.

MATERIALS AND METHODS

Mosquito specimens - A total of 4,893 mosquitoes were collected in four departments in Colombia (Antioquia, Amazonas, Caquetá and Norte de Santander) in 2006 as part of the PhD study of Ruiz (2010). Of 574 mosquitoes collected in Amazonas, where *An. nuneztovari* s.l. is unreported, two specimens were keyed out as *An. nuneztovari* s.l. using the morphological keys of Suárez et al. (1988) and Faran (1980). DNA was extracted

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TABLE

Field codes, collection localities and co-ordinates of specimens of *Anopheles dunhami* and *Anopheles nuneztovari* C used in this study, alongside GenBank accessions for second internal transcribed spacer (ITS2) and cytochrome c oxidase I (COI) sequences where appropriate outgroup taxa were chosen based on their close morphological similarity to *An. dunhami*

Species	Country, locality (co-ordinates)	Field code	GenBank ITS2	GenBank COI
An. dunhami	Brazil, Amazonas, Tefé	SA26	HQ020394	-
	(-03.3207, -64.7235)	SA44	HQ020395	HQ315878
		SA79	HQ020396	HQ315879
		SA85	HQ020397	HQ315880
		SA98	HQ020398	HQ315881
	Colombia, Amazonas, Leticia	C08921	HQ020382	-
	(-04.1158, -69.9522)	C09041	HQ020383	HQ315867
An. nuneztovari C	Colombia, Antioquia, Zaragoza	Za-106	HQ020399	-
	(07.4847, -74.8679)	Za-110	HQ020400	-
		Za-130	HQ020401	-
		Za-131	HQ020402	-
		Za-158	HQ020403	-
		Za-207	HQ020404	-
		Za-208	HQ020405	-
	Colombia, Norte de Santander, Tibú	NS-17	HQ020388	HQ315872
	(08.6403, -72.7372)	NS-62	HQ020391	HQ315875
		NS-71	HQ020392	HQ315876
		NS-72	HQ020393	HQ315877
		NS-130	HQ020384	HQ315868
		NS-135	HQ020385	HQ315869
		NS-139	HQ020386	HQ315870
		NS-144	HQ020387	HQ315871
		NS-243	HQ020389	HQ315873
		NS-319	HQ020390	HQ315874
Outgroups				
Anopheles oswaldoi B	Colombia, Antioquia, Nechí (08.1100, -74.7670)	CO9000	-	HQ315866
Anopheles rangeli	Brazil, Mato Grosso (-10.2256, -54.9861)	BR704-40	-	HQ315865
Anopheles trinkae	Ecuador, Napo, Sardina Yacu	SY205	-	HQ315882
	(-00.1666, -77.0833)	SY227	-	HQ315883

from these two specimens and a further 17 individuals of *An. nuneztovari* from the departments of Zaragoza (n = 7) and Norte de Santander (n = 10) (Table). Given the morphological similarities between *An. nuneztovari* and *An. dunhami* and the reported presence of the latter species in the Brazilian Amazon, DNA was also extracted from abdomens of five archive specimens of *An. dunhami* collected at the type locality (Tefé, Brazil). Voucher

specimens are stored in the mosquito collections of the Smithsonian Institution of National Museum of Natural History (NMNH), Washington DC, USA (Table).

Molecular methods - DNA template was acquired using the commercially available DNeasy Blood & Tissue Kit and BioSprint 96 DNA (both QIAgen®, Maryland, USA). Amplification of rDNA ITS2 polymerase chain reaction (PCR) was carried out using the primers of Collins and Paskewitz (1996) and the protocol described by Linton et al. (2001).

Amplification of the 710 bp fragment of the barcoding region of the mitochondrial COI gene was achieved using the primer pair LCO 1490 and HCO 2198 developed by Folmer et al. (1994), using a PCR protocol optimized by the Laboratories of Analytical Biology, Museum Support Center, Smithsonian Institution/NMNH. Each PCR contained 1 x NH_4 buffer, 0.5 mM each dNTP, 2 mM of MgCl, 0.2 U of Taq polymerase (BioLine, Taunton, MA, USA), 0.3 µM each primer and 1 µL of DNA template and was made up to a total volume of 10 µL using ddH_20. The PCR thermocycler parameters included: a single cycle at 95ºC for 5 min, followed by 34 cycles of 95ºC for 30 s, 48ºC for 30 min and 72ºC for 45 s, respectively, terminating with a 72ºC for 5 min extension step and a 10ºC hold. PCR products (both ITS2 and COI) were visualized on 1% agarose gels, containing 0.5 mg/mL of ethidium bromide and purified using ExoSAP-IT® (USB Corporation, Cleveland, Ohio, USA).

Sequencing reactions were carried out in both directions using the Big Dye Terminator Kit® (PE Applied BioSystems, Warrington, England) on an ABI 3730 automated sequencer (PE Applied BioSystems). Sequences were edited using Sequencher™ v.4.8 (Genes Codes Corporation, Ann Arbor, MI) and either aligned automatically in ClustalX (Thompson et al. 1997) or manually using MacClade v.4.06 (Maddison & Maddison 2003). Sequence similarities were compared with those available in GenBank using Basic Local Alignment Search Tool (blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence divergence was calculated in MEGA version 4 (Kumar et al. 2008) using the Kimura 2-parameter distance model (K2P) (Kimura 1980). A bootstrap neighbor joining (NJ) tree (Saitou & Nei 1987) was generated in PAUP version 4.0 (Swofford 2003), using 1,000 replicates of the K2P distance matrices.

COI sequences generated formed part of the activities of the Mosquito Barcoding Initiative (project leader YML) and both full specimen and COI sequence data, including electropherograms, are freely available through the Barcode of Life database website at boldsystems.org. GenBank accession for sequences generated in this study are listed in Table.

RESULTS

ITS2 sequences (n = 5) and COI DNA barcodes (n = 4) were generated from five topotypic archive specimens of *An. dunhami* from Tefé. ITS2 sequences from 19 Colombian *Anopheles*, morphologically identified as *An. nuneztovari* using the keys of Faran (1980) and Suárez et al. (1988) revealed two distinct ITS2 sequences, differing at 17 bases [1 transition (G/A), 1 transversion (C/G) and 15 indels] (Fig. 1).

The most frequent ITS2 sequence was shared by seven specimens from Zaragosa (Antioquia) and 10 specimens from Tibú (Norte de Santander), morphologically identified as *An. nuneztovari* s.l., whereas a different sequence was shared by two specimens collected in Kilómetro 12, Leticia (Amazonas, Colombia). The most frequent sequence was identical to previously published sequences of *An. nuneztovari* C from Colombia (Sierra et al. 2004) (AY028081-124, AY028128), Brazil (Marrelli et al. 2005) (AF461749) and Venezuela (Fritz et al. 1994) (L22462).

The two specimens from Kilómetro 12, Leticia showed 100% identity with topotypic *An. dunhami* sequences and three published *An. dunhami* sequences from Parintins (EU848337, EU848338 and EU848344) (Calado et al. 2008). However, sequence differences were noted between the topotypic and Colombian specimens and the remaining six available *An. dunhami* sequences (generated from clones) reported from Parintins. Topotypic and Colombian *An. dunhami* shared 99.7% identity with EU848340 and EU848341 (1/360 variant bases), 99.4% identity with EU848339, EU848343, EU84845, same haplotype (2/360 variant bases) and 98.9% with EU848342 (4/360 variant bases) (Fig. 1).

MtDNA COI DNA barcode sequences (658 bp without primers) were generated for specimens previously identified by ITS2 sequence data as follow: topotypic *An. dunhami* from Brazil (n = 4) and Colombia (Kilómetro 12, Leticia) (n = 1) and *An. nuneztovari* C from Norte de Santander (n = 10). COI barcode data were also generated for *An. trinkae* (n = 2), *An. oswaldoi* B (n = 1) and *An. rangeli* (n = 1), as these species can be morphologically mistaken for *An. dunhami* or *An. nuneztovari* s.l. in the female adult stage (Faran 1980).

The bootstrap NJ tree (Fig. 2), based on 1,000 replicates of the K2P COI distance matrices (Kimura 1980), reaffirmed the specific identity of topotypic and Colombian *An. dunhami* and its genetic differentiation from other closely related taxa including *An. nuneztovari* C, *An. trinkae*, *An. oswaldoi* B and *An. rangeli* obtained by ITS2. Mean intra-specific K2P

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                                                  1222222222233333333
                                                  2922478888890112255555
                                                  4923390123408454534 56
An. dunhami (n=5), Bra., Tefé                     t---aagagagaaga------
An. dunhami (n=2), Col., Amazonas                 .---.....-----
An. dunhami (EU848337-38,44), Bra., Par.          .---.....-----
An. dunhami (EU848339,43,45), Bra., Par.          .---.....--...-----
An. dunhami (EU848340), Bra., Par.                .---g.....-----
An. dunhami (EU848341), Bra., Par.                .---.....g-----
An. dunhami (EU848342), Bra., Par.                c---.....--c..-----
An. nuneztovari (n=17), Col., N.S., Zar. .ggg.------.gc.tgagag
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Fig. 1: alignment showing differing bases of second internal transcribed spacer sequences of Colombian *Anopheles dunhami* (546 bp; n = 2) and *Anopheles nuneztovari* C (549 bp; n = 17) generated in this study in comparison to topotypic *An. dunhami* from Tefé, Brazil (546 bp; n = 5) and other available sequences available in GenBank (360 bp; EU848337-42) (Calado et al. 2008). Bra: Brazil; Col: Colombia; Par: Parintins; N.S.: Norte de Santander; Zar: Zaragoza.

divergence values were 0.007, 0.004 and 0.010 in *An. dunhami* (n = 5), *An. nuneztovari* C (n = 10) and *An. trinkae* (n = 2), respectively. The mean K2P value between *An. dunhami* and *An. nuneztovari* was 0.025, *An. dunhami* and *An. trinkae* was 0.093, *An. nuneztovari* and *An. trinkae* was 0.085.

DISCUSSION

This study provides DNA sequence data for topotypic specimens of *An. dunhami* for the first time, thus establishing the genetic identity of the species. Correlation of the ITS2 rDNA and COI barcodes of Colombian specimens from the Colombian Amazon with topotypic *An. dunhami* confirms the first record of *An. dunhami* outside Brazil and comprises a new record for Colombia. That *An. dunhami* can be morphologically misidentified with vector species, including *An. nuneztovari* s.l. (as in this study), *An. trinkae* and *An. oswaldoi* s.l., is of significance to vector control personnel in Colombia, as it could result in wasted resources, with unnecessary control measures mistakenly being applied against this non-vector species.

Topotypic ITS2 sequences of *An. dunhami* were less than 1% dissimilar to those reported by Calado et al. (2008). The two remaining GenBank entries labelled as *An. dunhami* are problematic: U9236 (Tefé) (Lounibos et al. 1998) and AF462378 (Acre, Brazil) (Marrelli et al. 2005). As highlighted by Calado et al. (2008), significant differences exist between the GenBank submission (U9236) and the published sequences in the paper of Lounibos et al. (1998) and the submitted sequence shows 15 base differences (all indels) from our *An. dunhami* sequences. An author of the present study (JEC) and co-author of Lounibos et al. (1998) confirms that manual sequencing errors are most likely to account for these discrepancies. GenBank entry AF462378 of Marrelli et al. (2005) was more similar (96%) to *An. oswaldoi* s.s. from Brazil, São Paulo (AF425915), than topotypic *An. dunhami* 86% (68/496 variant bases).

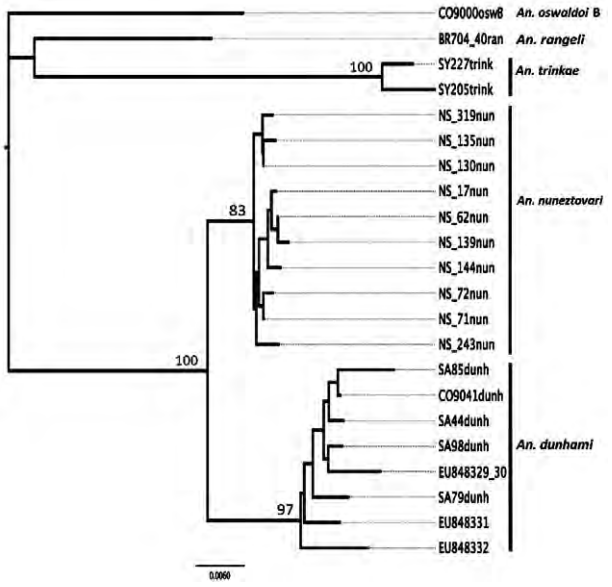

Fig. 2: bootstrap neighbor-joining tree constructed using 1,000 replicates of Kimura two-parameter distance model genetic distance matrices of cytochrome c oxidase unique haplotypes (680 bp) belonging to 23 specimens (only unique haplotype are shown) of *Anopheles nuneztovari* [Norte de Santander, Colombia (n = 10), GenBank HQ315868-HQ315877], *Anopheles dunhami* [Kilómetro 12, Amazonas, Colombia (n = 1), HQ315867; Tefé, Amazonas, Brazil (n = 4), HQ315878-HQ315881; EU848330; EU848330 (same haplotype as EU848329), EU848331, EU848332 (Calado et al. (2008)]. Out-groups: *Anopheles trinkae* (Sardina Yacu, Napo State, Ecuador, HQ315882-HQ315883), *Anopheles oswaldoi* B (HQ315866) and *Anopheles rangeli* (HQ315865).

Although the two *An. dunhami* specimens detected were field identified by females as *An. nuneztovari* s.l., these taxa are easily differentiated using either ITS2 or COI sequence data or male genitalia (Calado et al. 2008). *An. dunhami* and *An. nuneztovari* C share only 96.9% identity at ITS2 (Fig. 1) and 97.5% at COI (Fig. 2). *An. dunhami* ITS2 sequences generated in this research share 78-80% identity with *An. trinkae* sequences in GenBank (U92346, U92355 and Y09075) (Fritz 1998, Lounibos et al. 1998).

Contrary to the low level of intra-specific COI sequence divergence, the mean inter-specific K2P distance between *An. dunhami*, *An. nuneztovari* and *An. trinkae* was 0.067 (range 0.025-0.093). Comparable results were reported in other barcode studies on mosquitoes, i.e. 0.058 between *Anopheles fluviatilis* and *Anopheles minimus* (Kumar et al. 2007) and 10.4% (range 0.2-17.2%) for nine genera of Culicidae (Cywinska et al. 2006). In contrast to the ITS2 dataset, using mtDNA markers, *An. dunhami* and *An. nuneztovari* appear most closely related with 2.5% sequence divergence, but significantly higher than intra-specific comparisons.

Herein we verified the presence of *An. dunhami* in Colombia for the first time using both ITS2 sequences and COI barcodes. Although *An. dunhami* has not been implicated in malaria transmission (Peyton 1993, Lounibos et al. 1998), adult females, the stage most readily collected in epidemiological studies, can be easily misidentified as *An. nuneztovari* s.l., *An. oswaldoi* s.l. or *An. trinkae*, all of which are proven natural vectors of malaria in South America (Faran 1980, Hayes et al. 1987, Herrera et al. 1987, Olano et al. 2001, Quiñones et al. 2006, Gutiérrez et al. 2009). It is possible that *An. dunhami* may also be a competent vector; however, its role in local malaria transmission is masked by the presence of other morphologically similar species, which are traditionally known as malaria vectors. Knowledge of mosquito species present in vector control regions is important. In Colombia, local vector control strategies vary according to the vector species present and this in turn is driven by the entomological surveys, thus it is now imperative that morphological keys in Colombia are updated to include *An. dunhami* and to determine novel morphological characters to differentiate this species from *An. nuneztovari* in the adult female stage if possible. It seems likely that this species will also be present in the Amazonian Region of Peru and that its eventual known distribution could include a large portion of the Amazon Basin. Topotypic ITS2 and COI sequences of *An. dunhami* from Tefé generated in this study will facilitate the identification of this species across its geographical range.

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